Penicillin and cephalosporin resistance in gonococci

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Abstract

Non-penicillinase producing *Neisseria gonorrhoeae* isolated at St Mary's Hospital, London were examined for the prevalence of resistance to penicillin and for decreased susceptibility to cefuroxime. Of the 941 non-PPNG tested 100 (10·6%) were resistant to penicillin (minimum inhibitory concentration, MIC, ⩾1 mg/l) and were considered to be chromosomally-resistant *N gonorrhoeae* (CMRNG). Decreased susceptibility to cefuroxime (MIC, ⩾0·5 mg/l) was detected in 79% of the CMRNG. The CMRNG were also more often prototrophic and of serogroup IB than the remaining non-PPNG. The correlation coefficient for resistance to penicillin and cefuroxime was high, 0·79. Transformation experiments with both genetically-defined strains and transformants obtained using DNA from clinical isolates, showed that increased resistance to cephalosporins was acquired in three steps in close association with penicillin. We think this suggests that the loci controlling resistance to the cephalosporins are identical or closely linked to those controlling penicillin resistance.

Introduction

Since 1980, strains of *N gonorrhoeae* exhibiting chromosomal resistance to penicillin (minimum inhibitory concentration (MIC), ⩾1 mg/l) have been isolated with increasing regularity. These strains have been reported worldwide including in the United Kingdom,[1] the Far East[2] and the United States.[3,4] In contrast to penicillinase-producing strains of *N gonorrhoeae* (PPNG), chromosomally-mediated resistant *N gonorrhoeae* (CMRNG) also show decreased susceptibility to cephalosporins,[5,6] although they are still therapeutically sensitive to these antibiotics. Strains of CMRNG are susceptible to spectinomycin. At the Praed Street Clinic, St Mary's Hospital, London CMRNG represented only 4% of strains tested in 1981 whereas this figure had risen to 6·6% in 1984.[5]

In *N gonorrhoeae*, chromosomally-mediated resistance to penicillin is non-enzymic and results from the additive effects of mutations at several distinct loci.[7–9] Mutation at the *penA* locus affects resistance to beta-lactam antibiotics only, while mutations at the *mtr* and *penB* loci are pleiotropic also increasing resistance to hydrophobic antibiotics and tetracycline respectively.[8] In addition two mutations, *pem* and *tem* have been identified which induce further increases in resistance to penicillin by modifying the expression of *penA* and *penA*, *mtr* and *tet* respectively.[9]

The mutations responsible for penicillin resistance such as *penA*[10] also confer decreased susceptibility to second and third generation cephalosporins although this has not been studied extensively.

We have reported previously a close association between chromosomal resistance to penicillin and decreased susceptibility to cefuroxime amongst clinical isolates of non-PPNG from the Praed Street Clinic.[5] We are concerned that widespread use of second and third generation cephalosporins for the treatment of gonococcal infection, although clinically justified, might exert selective pressure leading to increased prevalence of CMRNG. To investigate this possibility we have studied the prevalence of penicillin and cephalosporin resistance among strains of non-PPNG isolated from patients attending the Praed Street Clinic for Sexually Transmitted Diseases. We have also examined the genetic basis of chromosomal resistance to penicillin and cephalosporins in these strains.

Materials and methods

Strains of N gonorrhoeae

Nine hundred and forty-one isolates of non-PPNG were examined, all from patients attending the Praed Street Clinic, St Mary's Hospital, London between 1984 and 1988. Twenty isolates of non-PPNG were collected each month, the first ten isolates from men and from women, which we have shown previously to be representative of the whole population.[11]

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Table 1 Characteristics of strains carrying known antibiotic resistance loci described by Sparling et al [8]

Strain	*Loci*	*Fold increase to penicillin†*	*Origin*
FA19	Wild type	—	Wild type
FA102	*penA*	4–8	Transformant FA48* × FA19
FA136	*penA, mtr*	26	Transformant FA48* × FA102
FA140	*penA, mtr, penB*	120	Transformant FA48* × FA136

*FA48 DNA from donor strain described by Sparling *et al* [8].
†Increase in resistance redetermined in our laboratory using techniques described in Methods section.

For analysis of the genetic basis of resistance the following strains were used. A series of genetically characterized reference strains of *N gonorrhoeae*, FA19, FA102, FA136 and FA140 were kindly provided by Dr PF Sparling. These contain specified antibiotic resistance loci which together with their origin is described in table 1.[8] These strains were derived from FA19 in transformation experiments with the donor FA48 which was a two-step mutant from FA19 selected for resistance to penicillin and streptomycin.[8] Two recipients were used that were fully sensitive to penicillin: strain FA19, described above, and a laboratory-adapted strain, H1, which has been used previously in this laboratory.[12]

For our transformation experiments, five donor strains were used, of which E43, H39 and M211 were clinical isolates from patients attending St Mary's Hospital and T132 and T143 were from St Thomas' Hospital, London, and kindly supplied by Professor I Phillips. Each clinical isolate was resistant to penicillin (MIC, $\geqslant 1$ mg/l) and showed decreased susceptibility to cefuroxime (MIC, $\geqslant 0{\cdot}5$ mg/l).

Clinical strains were isolated on a selective medium consisting of GC agar base (BBL), 40 g/l, supplemented with 1% IsoVitaleX (v/v), vancomycin, 3 mg/l, colistin, 1000 units/ml, trimethoprim, 5 mg/l and amphotericin, 1·5 mg/l. After inoculation in the clinic, media were incubated for 48 hours at 36°C in 6% carbon dioxide. Isolates were identified presumptively as *N gonorrhoeae* on the basis of Gram stain and oxidase reaction. This was confirmed by utilisation of glucose, but not of maltose or sucrose and the failure to produce beta-galactosidase. Penicillinase production was determined by the chromogenic cephalosporin test.[13] Strains of PPNG were not included in this study. All strains were stored in 15% (v/v) glycerol broth in vapour phase liquid nitrogen (−135°C).

Susceptibility testing

Susceptibility testing of the 941 clinical isolates was performed using Diagnostic Sensitivity Test (DST) agar (Oxoid) supplemented with 10% (v/v) lysed horse blood (Tissue Culture Services) and 1% (v/v) IsoVitaleX (BBL). In addition we used GC agar base, 36 g/l (Difco) supplemented with 1% (v/v) IsoVitaleX (BBL) for transformation experiments and for determination of the susceptibilities of the transformants to allow comparison with published data.[8]

Susceptibility testing was performed using an agar dilution technique described previously.[5] The inoculum was 10^5 colony forming units (cfu) and the media were incubated at 36°C in 6% carbon dioxide for 48 or 24 hours for DST agar and GC agar respectively. The MIC was read as the lowest concentration of antibiotic to give complete inhibition of growth.

The majority of antibiotics used were in the form of Adatabs (Mast Laboratories). These included penicillin (4·0–0·008 mg/l), cefuroxime (4·0–0·008 mg/l), cefotaxime (0·25–0·001 mg/l), tetracycline (8·0–0·03 mg/l), fusidic acid (4·0–0·03 mg/l) and erythromycin (4·0–0·03 mg/l). Ceftriaxone (0·25–0·001 mg/l, Roche), was also used.

Auxotyping and serotyping

The nutritional requirements of the isolates were determined using the chemically defined medium described by Copley and Egglestone.[14] Strains were tested for their requirement for proline, arginine, hypoxanthine, uracil, methionine and histidine. The serovar was determined by coagglutination using a panel of monoclonal antibodies raised to the major outer membrane protein, PI, of *N gonorrhoeae* and bound to *Staphylococcus aureus* and using the method described by Knapp *et al*.[15]

DNA preparation

DNA was purified from penicillin resistant strains according to a method adapted from that of Sparling.[16] For each strain used, the overnight growth from 40 GC agar plates was suspended in saline-citrate buffer (0·15 M sodium chloride, 0·015 M sodium citrate). Sodium dodecyl sulphate (SDS) was added to a final concentration of 5% (w/v) and the suspension stirred for three hours at room temperature. Addition of two volumes of ice-cold 95% (v/v) ethanol yielded a fibrous precipitate which was dissolved in 1 M sodium chloride and centrifuged at 38,000 × *g* for one hour at 4°C. The supernatant was removed and the crude DNA fibres were precipitated using 95% (v/v) ethanol, washed in 75% (v/v) ethanol and dissolved in saline-citrate buffer.

The fibres were deproteinised twice by addition of

0·4% (w/v) SDS followed by stirring for three hours at room temperature. After each deproteinisation, the concentration of sodium chloride was raised to 1 M and the preparation centrifuged at 38,000 × *g* for one hour at 4°C. Fibres were precipitated and washed as above and suspended in saline-citrate buffer.

Contaminating RNA was removed by addition of pre-heated RNase (Sigma; 80°C for 10 minutes) to give a final concentration of 50 μg/ml and incubated at 37°C for 30 minutes. The DNA was quantified using the diphenylamine reaction.[17]

Transformation procedure

The method used was a modification of that described by Janik *et al.*[18] Piliated colonies of the recipient strain were suspended to a concentration of approximately 10^8 cfu/ml in proteose peptone (15 g/l, Difco). The degree of piliation was determined using colonial morphology when examined by a low power plate microscope. Equal volumes of the recipient and DNA were mixed on the surface of a GC agar plate. Limiting concentrations of DNA (final concentration < 1 ug/ml) were used throughout this study, having been determined previously by DNA titration experiments.[16] The transformation mixture was incubated for 30 minutes before the addition of 100 μg DNase (Sigma). The incubation was continued for a further six hours before the growth was suspended in saline and inoculated onto selective media, GC agar supplemented with penicillin at four times the MIC of the recipient. After 72 hours incubation, twenty transformant colonies were picked onto GC agar without antibiotics and subcultured once before their susceptibility profiles were determined. Experiments were controlled firstly by plating the untreated recipient directly onto selective media and secondly by incubating with DNA pretreated with DNase. The transformation experiments were repeated using a piliated transformant from the first level experiment which was re-exposed to the original donor DNA. Second-level transformants were selected on agar containing penicillin at the MIC of the new recipient. Third-level transformants were obtained in a similar manner but it was not possible to obtain transformants after a fourth exposure to the DNA.

Statistical analysis

Differences between the distribution of auxotypes and serogroups amongst CMRNG and the remaining non-PPNG were determined using chi square and Fisher's exact test.

Results

One hundred (10·6%) of the 941 non-PPNG studied had penicillin MICs of ⩾1 mg/l and were, therefore, classed as CMRNG. There were some striking differences between the biological characteristics of these 100 isolates and the remaining 841 non-PPNG. The majority (66%) of CMRNG were prototrophic, 20% required proline, 2% arginine, hypoxanthine and uracil (AHU) and 4% arginine for growth. In contrast, the remaining 841 non-PPNG showed a significantly different distribution between these auxotypes ($p = < 0{\cdot}001$). Only 40% (337) were prototrophic and 15% (128) proline-requiring, while 18% (148) were AHU-requiring and 12% (95) arginine-requiring. Ninety per cent of CMRNG belonged to serogroup IB and only 10% to serogroup IA whereas only 69% of the non-CMRNG strains belonged to serogroup IB and 31% to serogroup IA ($p < 0{\cdot}001$). Of the individual serovars, the most striking difference was seen in the distribution of serovar IB-5/7. Eighteen per cent of CMRNG were IB-5/7 while only 3% (25) of non CMRNG belonged to this serovar ($p = < 0{\cdot}001$).

Susceptibility to penicillin in the total population showed a bimodal distribution. In addition to the 100 (10·6%) CMRNG, 523 (55·6%) of isolates showed intermediate susceptibility to penicillin (MIC, 0·12–0·5 mg/l) and only 318 (33·8%) were fully susceptible (⩽0·06 mg/l). While only 98 (10·4%) of all non-PPNG had reduced susceptibility to cefuroxime (MIC, ⩾0·5 mg/l), 79% of CMRNG did so. Correlation coefficients for susceptibility to penicillin, cefuroxime, tetracycline, erythromycin and spectinomycin among the total population are shown in table 2. The closest correlation was between the two beta lactam antibiotics, cefuroxime and penicillin. The least correlation was between spectinomycin and the other antibiotics.

Having established that there was a high prevalence of CMRNG and a close association between penicillin and cefuroxime resistance in our population, we then carried out a series of transformation experiments to define the genetic basis of this associa-

Table 2 Correlation coefficients between penicillin, cefuroxime, tetracycline, erythromycin and spectinomycin of 941 nonPPNG

	Penicillin	*Cefuroxime*	*Tetracycline*	*Erythromycin*
Cefuroxime	0·79			
Tetracycline	0·47	0·42		
Erythromycin	0·47	0·56	0·38	
Spectinomycin	0·16	0·16	0·15	0·29

tion. DNA from donors E43 and M211 were used with recipient strain FA19 and DNA from donors H39, T132 and T143 with recipient strain H1. Three levels of transformants were obtained using penicillin selection. The levels of susceptibility to beta-lactam antibiotics of the genetically characterized strains, one donor, E43 and its transformants are shown in table 3. Both sets of strains were isogenic with the recipient FA19. The characterised strain FA102 showed a four-fold increase in resistance to penicillin in comparison to its recipient and FA136 and FA140 both showed further increases resulting in an eight- and 32-fold increase in MIC respectively. The transformants from the clinical isolate also showed a similar stepwise decreased susceptibility to penicillin.

The susceptibility of the FA series of strains when tested in our laboratory differ slightly from those published by Sparling *et al.*[8] This is probably due to variations in the technique such as size of inoculum and different batches of base medium. The susceptibility to the cephalosporins, cefuroxime, cefotaxime and ceftriaxone was also decreased in each of the FA strains and the transformants in a similar pattern to penicillin. The increase in MIC differed with the activity of the cephalosporin used, the largest increases were to cefuroxime, the least active and the smallest to ceftriaxone, the most active of the cephalosporins.

The results of susceptibility testing of the same strains to erythromycin, fusidic acid and tetracycline, in addition to penicillin, are shown in table 4. The characterised strain FA102, which has the mutation *penA*, showed an increase in the MIC of penicillin. There was also a two-fold increase in resistance to erythromycin and fusidic acid which was not reported by Sparling *et al.*[8] We consider that a difference of one dilution is not significant because of the nature of susceptibility testing. Strain FA136 showed an increase in the MIC of penicillin, erythromycin and fusidic acid, which is consistent with the presence of the mutations *penA* and *mtr*. Strain FA140 showed a further increase in MIC of penicillin but no increase to tetracycline which would have confirmed the presence of *penB* in addition to *penA* and *mtr*. However, the serovar of FA140 is IB-3 in comparison with that of FA19, FA102 and FA136 which is IA-2. This change in serovar suggests acquisition of the *por* (syn. *nmp*) locus which encodes for the major outer membrane protein, PI, which is closely linked to *penB*. *por* and *penB* have a co-transformation frequency of 98%.[19] Our failure to detect the increase in MIC of tetracycline that Sparling *et al*[8] found in these strains was probably due to differences in susceptibility testing methods rather than the absence of the locus.

Only third level transformants from the clinical isolate E43 showed increases in the MICs of erythromycin, fusidic acid and tetracycline, in addition to the penicillin MIC. These third level transformants

Table 3 Susceptibility to beta-lactam antibiotics for genetically characterised strains (FA19, recipient, FA102, FA136, FA140) the donor (E43) and the transformants (E43–1, –2, –3)

	MIC (mg/l)			
Strain	*Penicillin*	*Cefuroxime*	*Cefotaxime*	*Ceftriaxone*
FA19	0·03	0·03	0·004	<0·001
FA102	0·12	0·06	0·008	0·002
FA136	0·25	0·25	0·015	0·004
FA140	1·0	0·5	0·03	0·008
E43	4·0	2·0	0·06	0·03
E43–1	0·25	0·06	0·015	0·008
E43–2	0·5	0·25	0·015	0·008
E43–3	1·0	0·5	0·03	0·015

Table 4 Susceptibility to penicillin, erythromycin, fusidic acid and tetracycline for the genetically characterised strains (FA19, recipient, FA102, FA136, FA140), the donor (E43) and the transformants (E43–1, –2, –3)

	MIC (mg/l)			
Strain	*Penicillin*	*Erythromycin*	*Fusidic Acid*	*Tetracycline*
FA19	0·03	0·5	0·12	0·5
FA102	0·12	1·0	0·25	0·5
FA136	0·25	4·0	1·0	0·5
FA140	1·0	4·0	1·0	0·5
E43	4·0	1·0	1·0	4·0
E43–1	0·25	0·25	0·12	0·5
E43–2	0·5	0·25	0·12	0·5
E43–3	1·0	1·0	0·5	1·0

also showed a change in serovar from IA-2 to IB-3 as described for the isogenic FA series of strains. Although the pattern of increased resistance exhibited by the transformants obtained from DNA from clinical isolates differed from those of the characterised strains, all five clinical isolates we have used as donors gave the same pattern to the antibiotics tested as donor E43.

Discussion

We have looked at the antibiotic susceptibility of non-PPNG isolated at St Mary's Hospital, London twice before in 1981[20] and 1984–85.[5] In 1981 and 1984–85, 4% and 6% respectively of strains examined were CMRNG. In the population described here the prevalence of CMRNG has risen to 10·6% and indeed CMRNG are now a greater problem in St Mary's Hospital than are PPNG. There has been a fall in the percentage of strains showing intermediate penicillin susceptibility from 64% in the 1984–85 study to 56% now, while the level of fully sensitive isolates has remained broadly the same. Between 1984 and 1988 the number of gonococci isolated annually at St Mary's Hospital fell by over 65%, but this drop has had little impact on the pattern of antibiotic susceptibility of the strains of *N gonorrhoeae* isolated.

We have commented previously upon the association between auxotype, serovar and antibiotic susceptibility in general and the particular association between IB-5/7 and resistance to penicillin, cefuroxime and other antibiotics found in this study.[21] Penicillinase-stable cephalosporins, first cefoxitin and cefuroxime and then cefotaxime and ceftriaxone were introduced to treat infections caused by PPNG. Any relationship between the control of chromosomal resistance to penicillins and that to cephalosporins will become increasingly important clinically as cephalosporins are used more widely to treat gonorrhoea caused by resistant strains. Cephalosporins have been used only rarely to treat gonorrhoea at St Mary's Hospital, yet we have seen a steady rise in the MIC of cefuroxime which matches the rise seen in the MIC of penicillin.

The close correlation between chromosomal resistance to penicillin and reduced susceptibility to cephalosporins which we describe has been noted by others.[6] We have demonstrated that this association occurs in the isogenic series FA19, FA102 (*penA*), FA136 (*penA, mtr*) and FA140 (*penA, mtr* and *penB*). This also confirms the previous findings of Dougherty *et al*[10] using a separate isogenic series of strains. In addition we have shown that DNA from five clinical isolates resistant to both penicillin and cefuroxime was able to transform sensitive recipient strains to increased resistance to both penicillin and a range of cephalosporins in a similar incremental fashion. The pattern of susceptibility to erythromycin and fusidic acid in the transformants from the second- and third- level does differ from that of the FA series. We are, at present, unsure whether this indicates the presence of mutations at different loci controlling penicillin resistance. One major difference is that a laboratory mutant strain was used for donor DNA by Sparling *et al*[8] and we have used donor DNA from clinical isolates. There are small differences in the incremental increases in the MICs of penicillin and that of cephalosporins between the clinical transformants and the isogenic strains. Whilst these might reflect minor differences in the genetic loci involved they could also be caused by variation in the technique of susceptibility testing. Small differences in the physico-chemical behaviour of penicillins and cephalosporins in relation to penicillin binding proteins and cell wall permeability barriers of resistant strains may also contribute to this. These results show that the genetic control of chromosomal resistance to penicillin is closely related if not identical to that of resistance to second and third generation cephalosporins. The use of penicillin will exert some pressure on strains to become more resistant to both. This is in addition to the selective pressure for cephalosporin resistance exerted by the widespread use of ceftriaxone. Full characterization of the mutations involved in cephalosporin resistance in gonococci will require analysis of penicillin binding proteins and of outer membrane structure in relation to cephalosporin permeability.

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